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Phenotypic Characterization of *ipaH*+ *Escherichia coli* Strains Associated with Yolk Sac Infection

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SUMMARY. Seventy-six *Escherichia coli* serotypes possessing the *ipaH* gene typical of enteroinvasive *E. coli* (EIEC) strains were characterized. Biochemical identification of our strains shows positive reactions for lactose fermentation (100% of strains), lysine decarboxylase (98.7% of strains) and motility (67.1% of strains), properties that do not correspond with those described to the EIEC group. The serotypes agree with an initial classification. In this, some common O antigens identified among *ipaH*+ strains were O2 ($n = 20$), OR ($n = 11$) and non-determined O? ($n = 10$). The O2:NM serotype was the most common. Sixty-six percent ($n = 50$) of the *ipaH*+ *E. coli* strains were colicin producers, of them, 26 (34%) produced Col V and other colicins, 13 (17%) produced colicins other than Col V, and 11 (14.5%) produced Col V only. Trimethoprim/Sulfa (72%), ampicillin (64.5%), enrofloxacin (55.3%), and ciprofloxacin (47.4%) were the major antimicrobial resistance frequencies observed. Twenty-five different multiresistance patterns were observed, where sixty-six strains (86.8%) were included. A MIC test showed that most of the strains were sensitive to low gentamicin and kanamycin concentrations, whereas most of the strains were resistant to tetracycline. An invasiveness assay showed that the predominant alterations caused to HEp-2 cells were changes in shape and staining, and in most of the specimens, a partial monolayer detachment was also seen. Fifteen strains invaded more than 30% of the monolayer cells, causing the formation of intercellular bridges or filipoidal-like protrusions. The results suggest the existence of specific clone complexes derived from EIEC strains adapted to the avian host. To our knowledge, this is the first study that demonstrates the presence of extraintestinal invasive *E. coli* (ExIEC) strains.

RESUMEN. Caracterización fenotípica de cepas *ipaH*+ de *Escherichia coli* asociadas con la infección del saco vitelino.

Se caracterizaron setenta y seis serotipos de *Escherichia coli* portadoras del gen *ipaH*, característico de las cepas de *E. coli* invasoras del intestino. La identificación bioquímica de estas cepas mostró reacciones positivas a la fermentación de la lactosa (100% de las cepas), lisina decarboxilasa (98.7% de las cepas), y motilidad (67.1% de las cepas), propiedades que no corresponden con aquellas descritas para el grupo de cepas de *E. coli* invasoras del intestino. Los serotipos concordaron con una clasificación inicial en la cual se identificaron algunos antígenos comunes entre las cepas *ipaH*+ tales como el antígeno O2 ($n = 20$), OR ($n = 11$) y el antígeno O no determinado ($n = 10$). El serotipo O2:NM fue el serotipo más común. El 66% ($n = 50$) de las cepas de *E. coli ipaH*+ fueron productoras de colicinas, de las cuales 26 (34%) produjeron Col V y otros tipos de colicinas, 13 (17%) produjeron colicinas diferentes a la Col V, y 11 (14.5%) produjeron únicamente Col V. La resistencia microbiana observada con mayor frecuencia fue contra trimetroprina/sulfa (72%), ampicilina (64.5%), enrofloxacina (55.3%), y ciprofloxacina (47.4%). Se observaron 25 patrones de resistencia múltiple en 66 (86.8%) de las cepas de *E. coli*. La prueba de concentración inhibitoria mínima mostró que la mayoría de las cepas fueron sensibles a concentraciones bajas de gentamicina y kanamicina mientras que la mayoría de las cepas fueron resistentes a la tetraciclina. La prueba de invasividad mostró que las alteraciones predominantes ocasionadas a las células HEp-2 fueron cambios en la forma y la tinción, y en la mayoría de los especímenes se observó un desprendimiento parcial de la monocapa de células. Quince de las cepas invadieron más del 30% de la monocapa de células, ocasionando la formación de puentes intercelulares o proyecciones de tipo filipoidal. Los resultados sugieren la existencia de complejos clonales específicos de cepas de *E. coli* invasoras del intestino adaptadas al huésped aviar. De acuerdo con la información que tenemos, este es el primer estudio que demuestra la presencia de cepas invasivas extraintestinales de *E. coli*.

Key words: *Escherichia coli*, yolk sac infection, invasiveness, antibiotic resistance, virulence factors

Abbreviations: APEC = avian pathogenic *Escherichia coli*; Col V = Colicin V; EIEC = enteroinvasive *Escherichia coli*; EPEC = enteropathogenic *Escherichia coli*; ETEC = enterotoxigenic *Escherichia coli*; ExIEC = extraintestinal invasive *Escherichia coli*; IpaH = invasion plasmid antigen H; LPS = lipopolysaccharide; MEM = Eagle's minimal essential medium; MIC = minimal inhibitory concentration; PBS = phosphate-buffered saline; YSI = yolk sac infection

Escherichia coli is a common and important bacterial pathogen that causes at least 5% of the mortality in poultry flocks (13,21). Consequently, these bacteria are responsible for significant economic losses to the poultry industry (4,72,81). Pathogenic serogroups of *E. coli* are ubiquitous in environments in which poultry are raised and can cause air sacculitis, pericarditis, peritonitis, salpingitis, synovitis, osteomyelitis, cellulitis, or yolk sac infections (YSI) (5,19,29,63,65,81).

Recently, *E. coli* strains pathogenic to avian species have begun to be grouped as avian pathogenic *E. coli* (APEC) (9,13,15,17,18,81). Aside from causing embryo mortality (25,26,35), some features of APEC strains include the production of colicin V (15,25,26,

Table 1. Origin and serotypes of the *E. coli* strains included in the study.

O group	*ipaH*+	Source[A] (H antigen-number)	Total
O2	20	Ha19 (H1-1; H?-1), Ha21 (NM-2), Bro (NM-16)	20
O6	1	Bre (H16-1)	1
O8	1	Ha21 (H21-1)	1
O9	1	Bre (H11-1)	1
O12	1	Bro (H31-1)	1
O19	2	Bro (NM-2)	2
O22	2	Bro (H4-2)	2
O69	1	Ha19 (H38-1)	1
O78	4	Bro (H9-3; H?-1)	4
O81	1	Bro (H31-1)	1
O84	5	Bro (H8-4, NM-1)	5
O103	1	Ha19 (H11-1)	1
O112	—	Ha19 (H19-2)	2
O146	1	Ha19 (H19-1)	1
O152	—	Bro (H6-2)	2
O155	1	Bro (H10-1)	1
O167	2	Ha19 (H4-3)	3
O168	6	Ha19 (NM-1), Bro (NM-1, H5-3, H?-1)	6
OR	10	Bro (H4-1, H5-2, H6-1, H9-3, H31-2, NM-2)	11
O?	8	Ha19 (H4-1), Bro (H4-1, H7-1, H8-3, H31-3, H51-1)	10

[A]Bre = breeder farm; Ha19 = hatchery 19th day; Ha21 = hatchery 21st day; Bro = broiler farm.

27,35,49,63,81), a polysaccharide capsule (17,18,49,81), toxins and cytotoxins (15,17,18,49), F1-type fimbriae (15,17,18,25,27,35, 49,63,81), possession of *iss* genetic element (15,25,26,35), temperature-sensitive hemagglutinin (Tsh) (15,17,18,25,26,49,63), aerobactin iron-sequestering systems (15,17,18,27,49,63,81), and resistance to the host complement (15,17,18,25,27,35,49,63). Antibiotic resistance profiles and specific serotypes have also been identified in *E. coli* strains that cause colibacillosis (17,18,27,35, 49,81). Kariuki *et al.* (40) mentioned that the APEC group mainly consists of EPEC (enteropathogenic *E. coli*) and ETEC (enterotoxigenic *E. coli*) serovars.

For many years, it was thought that *E. coli* strains associated with YSI cases were avirulent or of low virulence (18,19,29). However, a recent study performed in our laboratory showed that 30% of the *E. coli* isolates recovered from breeder farm, hatchery, and broiler farm samples carried the *ipaH* gene, which has been associated with enteroinvasive *E. coli* (EIEC) strains (70). This observation suggests that invasiveness could play an important role in the development of YSI. EIEC strains are principally associated with human and primate infections, and their participation in the avian diseases is unknown. In this study, different properties of *ipaH*+ strains, such as serotype, colicin production, antimicrobial susceptibility, and ability to invade cell culture were analyzed in order to determine the characteristics that the strains implicated in the development of yolk sac infection possess.

MATERIAL AND METHODS

Strains. Seventy-six strains previously isolated from putative YSI cases (71) were analyzed. In a previous study (70), the strains were serotyped and analyzed for virulence factors. The *ipaH* gene-positive strains ($n = 68$) and eight more belonging to reported EIEC serogroups (43,44,52,76,83) were analyzed in this study (Table 1).

Strains were recovered from Dorset media (where they had been stored) and cultured onto McConkey and blood agar (Difco Laboratories, Detroit, MI). The obtained cultures were tested in the VITEK® AutoMicrobic System (Vitek AMS; bioMérieux Vitek, Marcy-l'Etoile, France) to confirm their identity.

Serotyping. The *ipaH*+ strains were newly typed to confirm their serotypes. Rabbit sera prepared against 175 somatic (O) and 56 flagellar (H) *E. coli* antigens (SERUNAM, Mexico City, Mexico), were used for typing as previously described by Orskov and Orskov (61). Additionally, 47 specific sera against *Shigella* strains and three more prepared in our laboratory against Mexican *E. coli* strains that do not react with the recognized antiserum scheme were used.

Colicin production. One of the most common characteristics of APEC strains is the production of colicin V. Consequently, a test for this trait was performed using the agar overlay method as previously described by Ramirez *et al.* (67). Briefly, strains were cultured overnight in tryptic soy broth at 37 C and plated in duplicate onto LTC agar, supplemented with yeast extract, tryptone, and calcium (1000 ml: 10 g tryptone, 5 g yeast extract, 10 g NaCl; pH 7.6 adjusted with 1M Tris; added with 50 mM $CaCl_2$ and 1% agar). Then the *E. coli* colonies were lysed by chloroform vapor exposure for 15 min. Thereafter, the plates were overlayed with 4.5 ml top soft LTC media (0.6% agar) containing 100 µl of an overnight growth of *E. coli* K12 711, a colicin indicator strain (sensitive to all colicins) or its isogenic mutant sensitive only to colicin V. The overlaid plates were allowed to grow overnight at 37 C and were then observed for the presence of an inhibition halo around colicin-producing colonies. Colicin V, colicin V and other colicins, colicins different from colicin V, and no-colicins producers are the four phenotypes that could be observed (45,67).

Antimicrobial susceptibility test. Antimicrobial susceptibility of the strains was determined using the automated VITEK® System for Antibiotic Susceptibility with GNS-100 No. V 4500 (bioMerieux Vitek) cards. The tested antimicrobials included in the card were amikacin, ampicillin, aztreonam, cefazolin, cefotaxime, cefoxitin, ceftazidime, ceftizoxime, ciprofloxacin, gentamicin, imipenem, nitrofurantoin, piperacillin, ticarcillin/clavulanic acid, tobramycin, and trimethopim/sulfa.

The minimal inhibitory concentrations (MICs) of gentamicin, tetracycline, and kanamycin were determined because these antibiotics are commonly used in local poultry farms and are not included in the VITEK® cards. Test concentrations of gentamicin were 100, 50, 25, 12.5, 6.25, 3.12, and 1.6 µg/ml; and 50, 25, 12.5, 6.25, 3.12, and 1.6 µg/ml for tetracycline and kanamycin. An inoculum of 3×10^5 CFU/ml, according to National Committee for Clinical Laboratory Standards (NCCLS) (53) was utilized in the assay. MICs were determined by the microdilution agar method using microtiter plates. Performance and evaluation of the MIC determinations followed the recommendations of the NCCLS (54). Ranges of susceptibility were noted along with the MIC that inhibited 90% (MIC90) of the isolates. Two reference strains (*E. coli* ATCC 25922 and *E. faecalis* ATCC 29212) were used for quality control in each set of plates. MIC breakpoints were $\geq$16 µg/ml, $\geq$64 µg/ml, and $\geq$16 µg/ml for gentamicin, kanamycin, and tetracycline, respectively. Determinations were made in triplicate.

Although enrofloxacin is one of the most common quinolones used in the Mexican poultry industry, it is not included in the VITEK® system card. Therefore, sensitivity of the strains to this antimicrobial was determined by the standard disk procedure described by Bauer *et al.* (6). The selection of disk concentrations (5 µg) and zone diameter interpretation was done as recommended by the manufacturer (Difco Laboratories, Detroit, MI) and by the NCCLS (53).

Invasiveness assay. The HEp-2 cell invasiveness test was performed as was described by Melhman *et al.* (48). Briefly, 2.5×10^6/ml of HEp-2 cells previously grown to confluence in Eagle's minimal essential medium (MEM) (GIBCO-BRL, Gaithersburg, MD), supplemented with 10% fetal calf serum, were plated onto coverslips on 24-well plates (Costar, Cambridge, MA) with fresh MEM, and incubated overnight at 37 C under a 5% CO_2 atmosphere. Simultaneously, *E. coli* strains were grown overnight in 5 ml of peptone water with D-mannose (3%). The bacterial cultures were centrifuged, washed, and resuspended in MEM without antibiotics. HEp-2 cells on plates were washed and

Table 2. Serogroup and colicin production of *ipaH+ E. coli* strains associated with yolk sac infection.

O group	No producers	Colicin-producing strains				Total strains
		Total	Col V	Col V and others	Others	
O2		20	4	15	1	20
O9		1			1	1
O19		2		2		2
O22		2	1		1	2
O69		1	1			1
O78	1	3	1	2		4
O84		5			5	5
O103		1		1		1
O112	2	—				2
O152		2			2	2
O167		3		3		3
O168	4	2		2		6
O various[A]	6	—				1
OR	6	5	2	1	2	11
O?	7	3	2		1	10
Total (%)	26	50 (65.8)	11 (14.5)	26 (34.2)	13 (17.1)	76 (100)

[A]O6, O8, O12, O81, O146, and O155, one strain per each serogroup.

Table 3. Antimicrobial susceptibility of *ipaH+ E. coli* strains associated with yolk sac infection.

Antimicrobial agent[A]	Resistance number (%)
Cefoxitin	1 (1.3)
Gentamicin	4 (5.3)
Ceftazidime	5 (6.6)
Nitrofurantoin	8 (10.6)
Cefazolin	11 (14.5)
Piperacillin	30 (39.5)
Ciprofloxacin	36 (47.4)
Ampicillin	49 (64.5)
Trimethoprim/Sulfa	55 (72.4)

[A]All the strains were sensitive to amikacin, aztreonam, cefotaxime, ceftizoxime, imipenem, tobramycin, Ticarcillin/Clavulanic Ac.

each well was refilled with 1 ml of fresh MEM without calf serum or antibiotics, but with 0.5% (wt/vol) of D-mannose. One hundred microliters of overnight-grown bacterial suspension containing 1.5×10^8 CFU were added to each well with HEp-2 cells. The plates were incubated for 3 hr in 5% CO_2 at 37 C. After this incubation, the cell cultures were washed three times with sterile phosphate-buffered saline (PBS). The wells were newly refilled with 1 ml of MEM containing gentamicin (100 μg/ml) and lysozyme (300 μg/ml) and incubated 3 hr more. After this incubation, the medium was pulled off and the cell preparations washed three times with sterile PBS, fixed with methanol for 10 min and Giemsa stained. The coverslips containing the stained HEp-2 cells were washed three more times with deionized water and dried with acetone, xylene–acetone, and xylene. The cells were fixed down with resin on the slips. The preparations were observed in a light microscope with the 100× objective. Positive (EIEC O28 ac) and negative (HB101 K-12) control strains were included in each assay.

Criteria for cytophatic effect include abnormal size or shape of mammalian cells, disruption of membrane, vacuolation, nuclear damage, and altered staining properties of nucleus and cytoplasm (48).

Two independent investigators, who were unaware of the origin of the isolates, examined all coverslips. The inspection of 10 fields routinely distributed in the preparation, each one containing between 15 and 30 cells, was carried out. A strain was considered invasive when at least 1% of the HEp-2 cells contained at least five bacteria in the cytoplasm (48).

RESULTS

Biochemical characterization. The 76 strains analyzed exhibited a positive lactose fermentation reaction, 98.7% were lysine decarboxylase positive through the VITEK® system, and 67.1% exhibited motility in semisolid medium.

Serotyping. The serotypes obtained in the previous study (70) were confirmed in this, although 12 of the strains previously identified as OR:NM in this study were serotyped as O2:NM. The relationship between the O antigens identified and *ipaH+* strains showed O2 ($n = 20$), OR ($n = 11$), and nontypable O? ($n = 10$) as the most common serogroups (Table 1). Considering the complete formula (O and H antigens), O2:NM was the predominant serotype isolated from specimens collected both from 21-day-old eggs at the hatchery and at the broiler farm. None of the serotypes isolated from specimens from the breeder farm were isolated from specimens collected during a later production stage (Table 1). Although nonmotile strains were the predominant group ($n = 25$), the most common identified flagellar antigens were H4 ($n = 8$), H8 ($n = 7$), and H31 ($n = 7$).

Some serogroups are traditionally associated with disease caused by the EIEC group; in this study, only O112 ($n = 2$), O167 ($n = 3$), and O152 ($n = 2$) were identified.

Colicin production. The colicin production assay showed four different patterns that were used to classify the analyzed strains. Fifty (66%) of the *ipaH+ E. coli* strains were colicin producers (Table 2). Of these, 34% (26/76) were Col V and other colicins positives, 13 (17%) produced colicins other than Col V and 11 (14.5%) produced only Col V.

No relationship between serogroup and colicin production was observed. However, all isolates of the O2 serogroup produced at least one type of colicin and most of these produced Col V and other colicins. Conversely, the O84 strains ($n = 5$) only produced colicins other than Col V (Table 2).

Antibiotic susceptibility. The VITEK® system revealed that all strains were sensitive to amikacin, aztreonam, cefotaxime, ceftizoxime, imipenem, tobramycin, and ticarcillin/clavulanic acid. However, 89.5% ($n = 68$) of the strains were resistant to at least one antibiotic. The major resistance frequencies were against trimethoprim/sulfa (72%), ampicillin (64.5%), and ciprofloxacin (47.4%) (Table 3). Conversely, a high number of enrofloxacin-resistant strains (55.3%) were identified.

Twenty-five different multiresistance patterns were observed among 66 of the 76 strains tested (86.8%) (Table 4). Only two isolates showed resistance against one antibiotic (one to ampicillin and the other against enrofloxacin); despite both belonging to the O2 serogroup, they were isolated from different sources (hatchery at 19th day and at broiler farm). Whereas resistance was observed in bacteria recovered from all the different stages of production, in only two cases were the same multiresistance patterns exhibited by a particular serotype isolated from more than one source. In these cases, resistance to enrofloxacin, ciprofloxacin, and trimethoprim/sulfa was shared by O2:NM isolated from both the hatchery at the 21st day and the broiler farm, and resistance to ampicillin, enrofloxacin, ciprofloxacin, piperacillin, and trimethoprim/sulfa was shared by O168:NM isolated from the hatchery at the 19th day and the broiler farm. Several serotypes collected from the broiler farm exhibited numerous multiresistance patterns and the widest multi–drug-resistant group (13 strains) was exclusively formed by O2:NM strains, which exhibited resistance to enrofloxacin, ciprofloxacin, and trimethoprim/sulfa. Serotype O?:H4 exhibited the

Table 4. Resistance patterns determined in *ipaH*+ *E. coli* strains associated with yolk sac infection.

Resistance patterns[A]	Number of strains
None	8
AM	1
ENO	1
AM, ENO	1
AM, PIP	3
AM, STX	5
ENO, CIP	4
ENO, STX	1
AM, ENO, CIP	2
AM, PIP, STX	7
AM, CZ, PIP	1
ENO, CIP, STX	13
AM, PIP, STX	1
AM, ENO, CIP, STX	4
AM, CZ, F/M, STX	1
AM, CZ, PIP, STX	2
AM, CZ, CAZ, F/M, STX	1
AM, CZ, CAZ, PIP, STX	2
AM, CZ, GM, PIP, STX	1
AM, CZ, F/M, PIP, STX	1
AM, ENO, CIP, GM, STX	1
AM, ENO, CIP, F/M, STX	1
AM, ENO, CIP, PIP, STX	9
AM, ENO, GM, F/M, STX	1
AM, CZ, CAZ, ENO, PIP, STX	1
AM, ENO, CIP, F/M, PIP, STX	2
AM, CZ, FOX, CAZ, ENO, GM, F/M, STX	1
Total	76

[A]AM = ampicillin; ENO = enrofloxacin; PIP = piperacillin; STX = trimethoprim/sulfa; CIP = ciprofloxacin; CZ = cefazolin; F/M = nitrofurantoin, CAZ = ceftazidime; GM = gentamicin; FOX = cefoxitin.

highest multiresistance pattern (eight antibiotics) and was isolated from the hatchery at the 19th day.

The gentamicin MIC test showed that 70 strains (92.2%) were sensitive to the lowest concentration of gentamicin, whereas only three of the strains were sensitive to 12.5 μg/ml. Seventy-five percent of the strains were sensitive to ≤1.6 μg/ml of kanamycin/ml and 23.7% ($n = 18$) sensitive to ≥50 μg/ml. In contrast, most of the strains were resistant to tetracycline: only 9.2% ($n = 7$) were inhibited with 3.12 μg/ml and 88.2% ($n = 67$) of the isolates were resistant to ≥50 μg/ml.

***In vitro* invasiveness assay.** Cytotoxic effects, including distortion of size or shape, disruption of membrane, vacuolation, nuclear damage, altered staining properties of nucleus and cytoplasm, and/or detachment of monolayer were seen in the HEp-2 cell preparations. The predominant alterations were the changes of shape and staining (Fig. 1a) compared with the negative control (Fig. 2). The percentage of cells infected by the different strains is presented in Table 5. A common feature in all the preparations was that the size of the bacteria observed in the HEp-2 cells was smaller than the positive control (Fig. 1a,b). Although in most of the specimens, a partial detachment was seen, 15 strains invaded more than 30% of the cells, causing the formation of intercellular bridges or filipoidal-like protrusions (Fig. 1b). In 10% of the preparations, the monolayer was completely detached.

DISCUSSION

EIEC and *Shigella* species are considered to be closely related (43,47,52,66,73,79,83,84) and, for some time, it has been clear that they could be placed in the same species (66). Both are generally lysine decarboxylase negative, nonmotile, and lactose negative (7,47,52,73,83). Our results showed that, despite carrying the *ipaH* gene characteristic of the EIEC group, our strains do not share the characteristics mentioned above.

Although only a few authors report EIEC strains able to decarboxylate lysine (30,47,76,83), our results showed that 98.6% ($n = 75$) of the studied strains were lysine decarboxylase positive. Regarding motility, 67.1% (43/51) of the *ipaH*+ strains were motile; this finding has been previously detected by some authors, who identified motile strains belonging to the EIEC group (7). In a study performed in Thailand (83), EIEC strains isolated from children with diarrhea were analyzed for lactose fermentation and the percentage of positive strains was higher (65%) than lactose-negative EIEC strains (35%); similarly, in the present work, all *ipaH*+ strains analyzed exhibited a positive lactose reaction. Therefore, the analyzed strains in the present study seem to belong to a different group than EIEC because they do not share these biochemical characteristics. Serotypes such as O28ac; O29, O42, O112ac; O124,

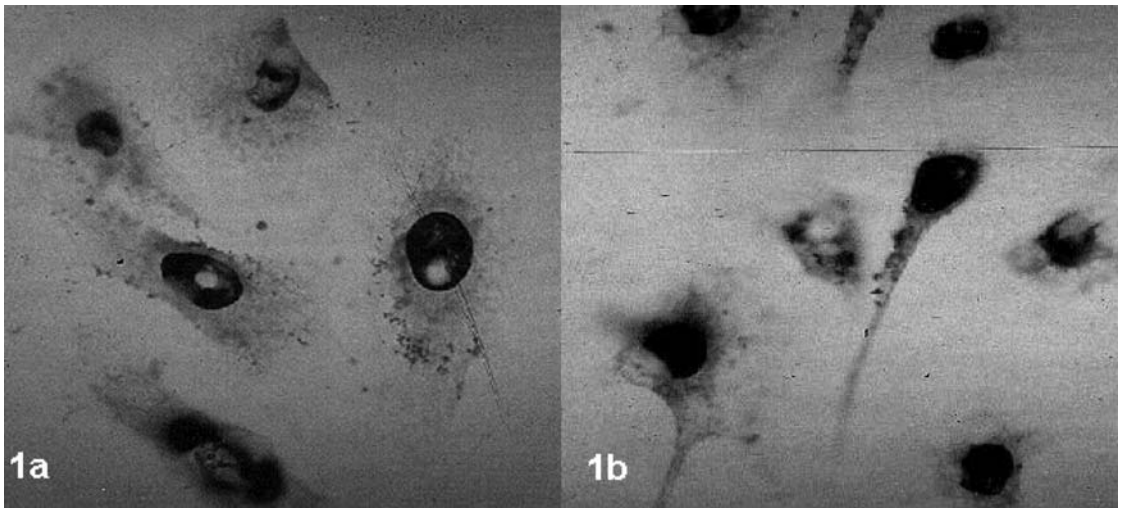

Fig. 1. (a) HEp-2 mammalian culture infected with *E. coli*. Host cells exhibit intracellular bacteria. (b) Filipoidal-like protrusions in HEp-2 cells in an *in vitro* invasiveness assay. Specimens were stained with Giemsa dye. Magnification 100×.

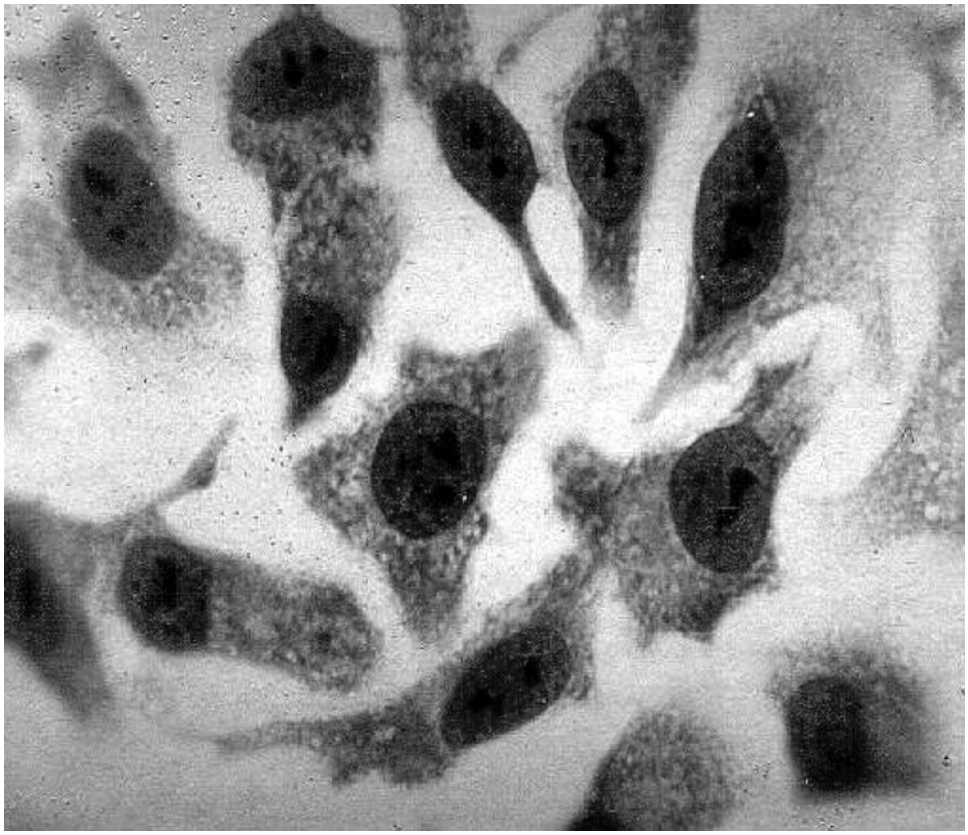

Fig. 2. Negative control of HEp-2 mammalian culture infected with *E. coli* (HB 101 K-12). Specimens were stained with Giemsa dye. Magnification 100×.

O136, O143, O144, O152, O159, O164, and O167 are considered as classical EIEC strains (7,43,44,52,76,79,83). However, in this study, only two of our *ipaH+* strains were included in those groups, and 96% belonged to other serotypes.

In poultry diseases, the serogroups most frequently identified are O1, O2, O78, and O untypeable (2,27,29,31,38,63,64). In the present study, O2 ($n = 20$) was the most common serogroup followed by OR ($n = 11$) and O? ($n = 10$). Barnes *et al.* (4) mentioned that most serotypes isolated from poultry are pathogenic only for birds; conversely, some O2 strains have been associated with urinary tract infections and meningitis in humans. Thus, the clonal relationship among *E. coli* strains is not necessarily reflected by their serotype (1,12).

It has been reported that humans and subhuman primates are the only natural hosts for EIEC strains (30,51). However, the *ipaH+* strains analyzed in the present study were isolated from poultry samples. This fact, along with the differences found in the biochemical and serotyping tests, suggests that our strains belong to specific clones that possess particular traits that make them able to cause disease in poultry.

Avian pathogenic *E. coli* strains have been grouped as APEC (9,13,15,17,18,81). However, omphalitis and yolk sac infection isolates frequently are not included in this group because some authors have mentioned that these strains are just opportunistic and nonpathogenic agents (18,19).

Several studies have shown that pathogenic avian *E. coli* strains produce colicins, which could be implicated in *in vivo* pathogenicity (8,16,20,21,25,27,35,63); but this trait is not shared by EIEC strains (22), whereas 66% of the *ipaH+* strains analyzed in this study were colicinogenic. Col V, an 88-amino-acid polypeptide (87), is one of the most common among 20 known colicins (86). It is found primarily among virulent bacteria implicated in extraintestinal infections in both humans and animals (44,78,86) and is related to the high mortality observed in the embryo lethality assay (27). This colicin could be considered as a virulence trait because it contributes to the elimination of competing microorganisms (3,11,65). Moreover, Col V was able to inhibit the growth of several Shiga-like-toxin–producing strains (37). Therefore, colicinogenic bacteria have an overall competitive advantage over colicin-susceptible wild-type bacteria, even if the colicinogenic bacteria are initially rare (3,25,34,42,62). On the other hand, the presence of Col V could indicate the presence of other virulence factors because Col V plasmids encode for other traits (36,44,81,86). In this study, 48.7% of the strains produced Col V, which is lower than the percentage of colicinogenic strains found in other reports (35,49,65,86), where colicin producers are between 72% and

Table 5. HEp-2 invasiveness assay in *ipaH+ E. coli* strains isolated from yolk sac infection.

% Host cell infected	No. of strains (%)
15–20	15 (19.7)
21–25	20 (26.3)
26–30	18 (23.7)
31–35	5 (6.6)
36–40	3 (4)
>40	7 (9.2)
MD[A]	8 (10.5)

[A]Monolayer detachment.

87%. However, our results agree with a similar study performed in Mexico (67).

Lior (44) mentioned that there is no correlation between serotype and colicin production because colicins are plasmid-encoded and potentially any strain could gain or lose these plasmids (44). In the present study, it was observed that most of the strains belonging to the O2 serogroup produced at least one colicin type, whereas O84 strains only produced colicins different from Col V.

Since 1950, control measures for *E. coli*-associated diseases have depended mostly on the prophylactic and therapeutic use of certain antibiotics. However, this practice has provided a selective pressure for antimicrobial resistance genes and, as a consequence, many bacteria associated with chickens and poultry meat are resistant to antimicrobial agents (5,14,24,40,55). The increasing resistance observed among avian strains has received considerable international attention (14,40) because they are a potential source of virulence genes that could be transmitted (4,12,58).

In the present study, 64.5% of the strains were ampicillin resistant. Besides the common and widespread use of ampicillin in the poultry industry, Bass *et al.* (5) presumed that the resistance against this antibiotic could be a reflection of cross-resistance to ceftiofur, a third-generation cephalosporin currently available as a therapeutic for poultry. In fact, we found resistant strains against other cephalosporins not currently used as therapeutic agents in avian medicine, such as cefazolin, ceftazidime, and cefoxitin. Resistance to two or more classes of antibiotics is now commonplace in both veterinary (33) and human medicine (5). An efficient route of acquisition and vertical and horizontal dissemination of resistance determinants is through mobile elements, including plasmids, transposons, and gene cassettes in integrons (68). Johnson *et al.* (36) analyzed the relationship between virulence and resistance in plasmids, finding that a 30-kb region contains genes encoding for ampicillin resistance; thus, these mobile components could be important in the pathogenesis and evolution of APEC strains.

Enrofloxacin, a fluorinated quinolone, was developed exclusively for use in animals, whereas ciprofloxacin is a potent antimicrobial agent used in human therapy (32,69). In the present study, high rates of resistance to both antimicrobials (55.3% and 47.4%, respectively) were observed. This finding could be due to a cross-resistance among these fluoroquinolones or because enrofloxacin is partially metabolized in the chicken liver to ciprofloxacin (32). Several studies have reported an increase in the resistance to quinolones; however, the percentages in those cases are generally low compared with the percentages observed in this study (12,55).

Several studies have shown that most of the isolated strains from avian colibacillosis are resistant to tetracycline and to different sulfonamides (5,12,14,40,58,63,81). Our results show a high number of resistant strains against tetracycline (88.2%). Moreover, several strains were resistant against antimicrobials not currently used in the poultry industry; similar findings have been reported previously and could be associated with transferable plasmids (14,36).

It has been documented that gentamicin resistance may be due to the inclusion of this antibiotic with the Marek's vaccine, administered to almost all poultry *in ovo* in other countries (5). In our study, most of the isolates (93.4%) were sensitive to this antibiotic. This result was important because this antibiotic is used in the invasiveness assay to eliminate bacteria that remain outside the cells, that is, noninvasive bacteria (50,57,82).

A relationship between virulence and resistance to antibiotics has been discussed recently (46). The development of resistance and virulence are both adaptive mechanisms acquired to enhance survival under stressful conditions (36). Therefore, the widespread dissemination of antibiotic resistance among bacterial populations could maintain or even increase the number of harmful bacteria involved in infections.

Of particular interest in our study was the high incidence of multiple-drug-resistant strains. Whereas only eight strains were sensitive to all the tested antibiotics (Table 4) and two were resistant to one antimicrobial, 87% ($n = 66$) were resistant to more than two antibiotics. The recovery of the multiple-drug-resistant strains from food animals has been widely documented (5,12,41,60). In particular, *E. coli* isolated from retail meats have been found resistant to frontline therapeutic antimicrobials, such as trimethoprim-sulphametoxazole, third-generation cephalosporins, and flouroquinolones (75).

Invasion is defined as a transfer of the agent from one cell to another of the same tissue or passage across a membrane barrier to infect another tissue. *Shigella* spp. and EIEC have the ability to invade guinea pig conjunctiva (Sereny test) (7,80). However, since the early 1970s, increasing attention has been focused on the use of cell culture assays (48,50) because they represent a uniform experimental system under which complex biological phenomena can be studied with defined conditions (57).

An initial report stated that it is necessary that a smooth somatic antigen be expressed for invasiveness to occur (23); however, Okamura *et al.* (59) concluded that oligosaccharides of both O antigens and rough cores of lipopolysaccharides (LPS) in *Shigella* spp. strains do not affect the ability of an invasive strain to penetrate tissue culture cells. Our observations support Okamura's work because all of our strains were positive for the invasiveness assay despite some of them ($n = 11$) expressing a rough LPS (OR strains).

Most of the expected cytopathic characteristics were seen in the evaluated preparations. A common feature among *ipaH*+ strains was the change of shape of the mammalian cells and altered staining properties, as well as disruption of the cell membrane (Fig. 1,b). Sometimes data could not be obtained because the monolayer detached from the slide during the infection phase ($n = 8$). Detachment produced by invasive strains may be attributed to cytotoxin production (48).

A remarkable result was that bacteria inside the cells were smaller than EIEC human strains; in fact, when a Gram-stained preparation was made from fresh cultures, the size of the bacteria was similar to those observed within mammalian cells. It is known that invasion phenotype is temperature regulated, requiring 37 C for growth (79,80). In fact, culture conditions, such as bacterial growth media, temperature, oxygen, growth state, can alter the assay (82). A possible explanation for the reduced size of the bacteria could be the source of isolation because our strains were recovered from poultry, for which body temperature is around 40 C. Obviously, APEC strains are adapted to cause damage under this condition, and the change of the environment could affect their size.

An important finding was that most of the analyzed strains produced extensions of the cytoplasm (Fig. 1b). These surface extensions are induced by *Shigella* spp. and EIEC (10,56,74,77,85). After the entry, the bacterium rapidly lyses the phagosomal membrane, multiplies freely in the cytosol, and, during this multiplication step, the bacteria can move intracellularly, leading to the formation of cellular protrusions that are driven by the bacterium and propel it into adjacent cells (10,28,48,56,74,85).

The current study has defined some characteristics of virulent *E. coli* strains associated with yolk sac infection and strongly suggest the existence of a limited number of clone complexes that possess particular traits that make them able to cause disease in poultry. Moreover, because most of our strains belonged to serotypes not previously reported as EIEC, this study provided the opportunity to study the role of specially adapted clones of extraintestinal-invasive

E. coli (ExIEC) strains able to be implicated in poultry diseases. To our knowledge, this is the first study that demonstrates the presence of ExIEC related to EIEC strains in a host different from primates, including humans. This information may be useful in improving our understanding of the pathogenesis of ExIEC infections. Further study is needed in order to establish the clonal relationship between avian ExIEC strains and classic EIEC and APEC strains.

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